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HPLC determination of chlorate metabolism in bovine ruminal fluid[§]

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Salmonella and *Escherichia coli* are two bacteria that are important causes of human and animal disease worldwide. Chlorate is converted in the cell to chlorite, which is lethal to these bacteria. An HPLC procedure was developed for the rapid analysis of chlorate (ClO_3^-), nitrate (NO_3^-), and nitrite (NO_2^-) ions in bovine ruminal fluid samples. Standard curves for chlorite, nitrite, nitrate, and chlorate were well defined linear curves with R^2 values of 0.99846, 0.99106, 0.99854, and 0.99138, respectively. Concentrations of chlorite could not be accurately determined in bovine ruminal fluid because chlorite reacts with or binds a component(s) or is reduced to chloride in bovine ruminal fluid resulting in low chlorite measurements. A standard curve ranging from 25 to 150 ppm ClO_3^- ion was used to measure chlorate fortified into ruminal fluid. The concentration of chlorate was more rapidly lowered ($P < 0.01$) under anaerobic compared to aerobic incubation conditions. Chlorate alone or chlorate supplemented with the reductants sodium lactate or glycerol were bactericidal in anaerobic incubations. In anaerobic culture, the addition of sodium formate to chlorate-fortified ruminal fluid appeared to decrease chlorate concentrations; however, formate also appeared to moderate the bactericidal effect of chlorate against *E. coli*. Addition of the reductants, glycerol or lactate, to chlorate-fortified ruminal fluid did not increase the killing of *E. coli* at 24 h, but may be useful when the reducing equivalents are limiting as in waste holding reservoirs or composting systems required for intense animal production.

Keywords: Bovine ruminal fluid; Chlorate; Chlorite; Food safety; Glycerol; HPLC; Nitrate; Nitrite; Sodium formate; Sodium lactate

Introduction

Salmonella and *Escherichia coli* are Gram (–) enteropathogens. *Salmonella* is an important cause of human and animal disease worldwide,^[1] and infection can cause serious illnesses or fatalities in the elderly and immunocompromised humans. The cost of medical care and lost productivity due to *Salmonella* infections in the United States were estimated at \$2.3 billion per year in 1998 dollars.^[2] The Centers for Disease Control and Preven-

tion (CDC) estimated that each year in the United States over 1.3 million human illnesses, over 15,000 hospitalizations and 553 deaths are caused by foodborne transmission of *Salmonella*.^[3] Pathogenic *E. coli* primarily cause three types of infections in humans: enteric, urinary tract, and septicemic infections.^[4] Among shiga-toxin-producing *E. coli*, strain O157:H7 is the most common.^[5] The CDC estimated that in the United States over 62,000 human illnesses, over 1,800 hospitalizations and 52 deaths are caused each year by foodborne transmission of *E. coli* O157:H7.^[3] The contamination of meat products with *E. coli* O157:H7 resulted in the recall of over 1 million pounds of meat in 2005.^[6] Over the decade prior to 2005, contamination of meat products with *E. coli* O157:H7 and *Salmonella* resulted in the recall of over 61.6 and 3.9 million pounds of meat products, respectively.^[6] Pathogen prevention strategies must be comprehensive and operative from farm-to-table,^[7] and some risk assessment studies predict that strategies to reduce the pathogen load in the live animal prior to slaughter would significantly reduce human exposure.^[8]

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A promising anti-pathogen strategy targets a biochemical mechanism common in Gram (–) enteropathogens. Intracellular reduction of chlorate by nitrate reductase (NR) is lethal to *E. coli* and *Salmonella*.^[9] *Salmonella*, like many members of the family Enterobacteriaceae, possess respiratory NR activity^[10] that can reduce chlorate to cytotoxic chlorite,^[11,12] whereas most gastrointestinal anaerobes lack NR and are not affected.^[13] It was hypothesized that chlorate may be used as a tool for pathogen reduction.^[14] The use of chlorate in veterinary and human medicine is not new,^[15] for example, chlorate salts maybe added to toothpastes at concentrations up to 5% as permitted by the European Union.^[15] However, the use of chlorate by the food industry in a preharvest pathogen reduction program is a novel concept.^[13]

Our laboratory has conducted *in vivo* food animal experiments to test this concept.^[14] Chlorate treatments were used in the drinking water of broiler chickens and significantly decreased *S. Typhimurium* in the crop contents,^[16] and *S. Typhimurium* levels were further reduced in broilers that were first nitrate-adapted and then treated with chlorate.^[17] Chlorate treatment via oral gavage of weaned pigs resulted in reduced cecal concentrations of *Salmonella*,^[13] and reduced *E. coli* O157:H7 in the pig gut.^[18] *Escherichia coli* O157:H7 in sheep were also reduced by chlorate supplementation.^[19,20] Chlorate application to bovine ruminal fluid *in vitro* lowered the levels of *E. coli* O157:H7 and *S. Typhimurium* DT104 from 10⁶ CFU to below the level of detection (≤ 10 CFU).^[14] *Escherichia coli* concentrations were significantly lowered in ruminal contents and feces of chlorate-treated cows compared to untreated cows.^[21] Chlorate treatment reduced *E. coli* and *E. coli* O157:H7 throughout the intestinal tract of cattle, but did not alter the total culturable anaerobic bacterial counts or the ruminal fermentation pattern.^[22] Chlorate treatment of feedlot cattle significantly reduced *E. coli* by as much as 100-fold in the gut and by up to 10-fold on the hide at the rump. The highest treatment levels (500 mg/kg body weight) showed no adverse effects during our short-term treatments.^[23,24]

Studies using [³⁶Cl[–]]-labeled chlorate administered to rats and cattle found that most of the labeled chlorine was present as chloride,^[25–28] and no evidence for the presence of chlorite was observed in any beef cattle tissues.^[28] Some researchers have suggested that since *E. coli* O157:H7 contamination of beef products remains a persistent problem, and since residue studies have shown that the vast majority of the chlorate-related residues in edible tissues were present only as the chloride ion, further research on chlorate as a preharvest intervention strategy was warranted.^[28] Studies are needed to examine potential chlorate, chlorite and nitrate residues in ruminal fluid, and the affect of these residues on ruminal bacteria. Chlorate had been determined in well water by a colorimetric method,^[29] in plasma and urine by a chlorate reductive method,^[30] in produce rinse water^[31] and drinking water^[32] by liquid chromatography, and residue studies were completed in beef cattle

and swine tissues by quantifying dosed radioactive sodium [³⁶Cl]chlorate.^[27,28,33] A method for quantifying chlorate and the other ions in a complex milieu such as bovine ruminal fluid is needed. The objective of this study was to develop a rapid, quantitative method for the analysis of chlorate, chlorite, nitrate and nitrite in ruminal fluid; to evaluate the loss of chlorate ion during aerobic and anaerobic incubation of chlorate-fortified ruminal fluid supplemented with added reductants that may affect the formation of chlorite and the killing of *E. coli*; and to determine the effect of chlorate on wildtype *E. coli* in ruminal fluid.

Materials and methods

Reagents and materials

Ethylenediamine, potassium dichloroacetate, potassium iodide, sodium chlorite, sodium nitrite, sodium thiosulfate volumetric standard solution, and starch indicator were obtained from Aldrich (Milwaukee, WI). Sodium chlorate and sodium nitrate were obtained from J. T. Baker (VWR Scientific Products, Houston, TX). Water (H₂O) used for dilution of standards and samples was produced on site by a reverse osmosis system obtained from Millipore Corp. (Bedford, MA), and was pyrogen-free. Water used for HPLC was double distilled.

Preparation of standards

Chlorite is susceptible to degradation by iron salts and chlorine, therefore, ethylenediamine (EDA) is used as a preservative for chlorite; EDA is also a recommended preservative for the determination of chlorate.^[32] We have observed that the addition of EDA to ruminal samples also results in cleaner samples for HPLC analysis (data not shown). The EDA working solution was prepared by diluting 2.81 mL (41.58 mmol) of 99% EDA with H₂O in a 25-mL volumetric flask, resulting in a 100 mg/mL solution of EDA. The final concentration of EDA in standards and samples was 500 μ g/mL. The chlorate (ClO₃[–]) ion standard was made by dissolving sodium chlorate, 62.78 mg (0.59 mmol), in a 100-mL volumetric flask with H₂O, resulting in a 500 μ g/mL solution of ClO₃[–]. Since high purity sodium chlorite is not commercially available, the simplest approach to determine the exact percent of NaClO₂ in the standard is to use the iodometric titration procedure.^[32] The chlorite (ClO₂[–]) ion standard solution was made by dissolving 44.3 mg NaClO₂ with 75.7% purity (0.37 mmol) (purity was determined by the iodometric titration method described below), in a 50-mL volumetric flask with the EDA working solution (250 μ L) and H₂O resulting in a 500 μ g/mL solution of ClO₂[–]. The nitrate (NO₃[–]) ion standard was made by dissolving sodium nitrate, 68.54 mg (0.806 mmol), in a 100-mL volumetric flask with H₂O, resulting in a 500 μ g/mL solution of NO₃[–]. The nitrite (NO₂[–]) ion standard was made

by dissolving sodium nitrite, 75 mg (1.087 mmol), in a 100-mL volumetric flask with H₂O, resulting in a 500 µg/mL solution of NO₂⁻. Dichloroacetate was used as an internal marker (IM), and was made by dissolving potassium dichloroacetate, 522.26 mg (3.13 mmol), in a 50-mL volumetric flask with H₂O, resulting in an 8 mg/mL solution of Cl₂CHCO₂⁻.

Iodometric method for chlorite determination

A titration, based on the Standard Method 4500-ClO₂ B; Iodometric Method^[34] and on the method of Aieta *et al.*^[35] was used to determine the level of ClO₂⁻ in the stock solution. Briefly, 2 mL of pH 7.0 phosphate buffer (25.4 g/L anhydrous KH₂PO₄, 64.4 g/L Na₂HPO₄) was added to a purge vessel. Distilled-deionized water (25 mL) and the sample (10 mL) were added. The solution was purged with argon for 15 min. using a gas-dispersion tube for good gas-liquid contact. Potassium iodide granules (1 g) were then added with stirring. If color develops at this point, the solution must be titrated to its end point with sodium thiosulfate (0.1 N). If titrated, the reading A = mL of titrant/mL of sample is recorded. To the same sample was added 3 mL of 2 M H₂SO₄. The mixture was allowed to react in the dark for 5 min., and then titrated with sodium thiosulfate (0.1 N) until the yellow color of the liberated iodine was almost discharged. Starch solution (1 mL) was added and the solution was titrated to the end point (blue color was discharged). The reading was recorded.

The values obtained from the titration are: A = Cl₂ (Only the portion not volatilized by the purge step. This value was not used in the calculation of ClO₂⁻); B = ClO₂⁻ (in mL of titrant/mL of sample); and N = Normality of the titrant used in equivalents per liter. Chlorite concentration (mg/L) of the sample was calculated as follows: [ClO₂⁻] = B × N (eq/L) × 16863 (mg/eq.)

High performance liquid chromatography (HPLC)

Chromatographic separation was carried out with a 4 mm internal diameter × 250 mm 9-µm IonPac[®] AS9-HC analytical column (Dionex Corp., Sunnyvale, CA) using 0.22-µm filtered (filter No. GSWP 047 00; Millipore Corporation, Bedford, MA) 9 mM Na₂CO₃ delivered at a flow rate of 1.0 mL/min. by a Dionex Gradient Pump (HPLC method according to manufactures recommendations, Dionex). The solvent system was degassed using helium. Samples containing chlorite were prepared in amber 11 mm crimp top auto-sampler vials (No. 12902; Pierce, Rockford, IL), and all other samples were prepared in clear 11 mm crimp-top auto-sampler vials (No. 12894; Pierce). The vials were sealed using crimp-top seals (No. 5181-1210; Hewlett Packard, Palo Alto, CA). Samples were introduced via a Spectra-physics SP8880 autosampler with an injection

volume of 25 µL. The column's effluent was monitored by a Pulsed Electrochemical Detector (Dionex).

Spiked ruminal fluid study

Ruminal contents were obtained from a cannulated Holstein cow maintained on a rye grass pasture. The freshly collected contents were withdrawn from the cannula and filtered through a nylon paint strainer^[36] and the resultant fluid was collected in an Erlenmeyer flask and returned to the lab within 1 h of collection. The ruminal fluid was centrifuged at 14,000 × g with a Sorvall[®] RC 5C Plus centrifuge (Kendro Laboratory Products, Newtown, CT). The supernatant was filtered through a 0.2-µm Acrodisc[®] syringe filter (Acrodisc[®] LC 13 mm Syringe Filter with 0.2-µm PVDF Membrane, PN 4455T; VWR Scientific Products, Houston, TX), and stored at -20°C. Chlorate, chlorite, nitrate, and nitrite ion standard solutions, EDA (5 µL) and IM solution (25 µL), were added to ruminal fluid and diluted with H₂O resulting in 1-mL samples containing 0.5, 1, 2, 3, 4, 5, 25, and 50 ppm ion concentrations with a final dilution of ruminal fluid of 1/10. These samples were filtered with a 0.2-µm Acrodisc[®] syringe filter prior to HPLC analysis.

Standard curves for the spiked ruminal fluid

Standard curves were generated by adding chlorate, chlorite, nitrate, and nitrite solutions, EDA (5 µL), potassium dichloroacetate (IM) solution (25 µL), and diluted with H₂O to a total volume of 1 mL to produce standards containing 0.5, 25, 50, 75 and 100 ppm of chlorate, chlorite, nitrate, and nitrite ions. Each sample was filtered through a 0.2-µm Acrodisc[®] syringe filter prior to HPLC analysis.

Disappearance of chlorate in anaerobic and aerobic ruminal fluid study

Ruminal contents were collected at approximately 08:00 am from a cannulated Jersey cow maintained on a rye grass pasture. The freshly collected contents were withdrawn from the cannula and filtered through a nylon paint strainer^[36] into a 1-L vessel. When completely filled, the vessel was capped and returned to the laboratory for immediate (within 30 min) anaerobic (100% CO₂) or aerobic distribution (10-mL volumes) to 160-mL crimp top culture vials, each having a diameter of approximately 50 mm. These vials were pre-loaded with small volumes of stock concentrations of chlorate (0.4 mL, 254 mM), and some vials also received 0.2 mL each of either sodium formate (1600 mM), sodium lactate (1600 mM), or glycerol (1600 mM). Vials were normalized to a final volume of 11 mL by addition of water achieving a final concentration of 770 ppm ClO₃⁻ in the samples. Vials loaded anaerobically were closed with rubber stoppers; those loaded aerobically were loosely covered with aluminum foil. The pH measured in

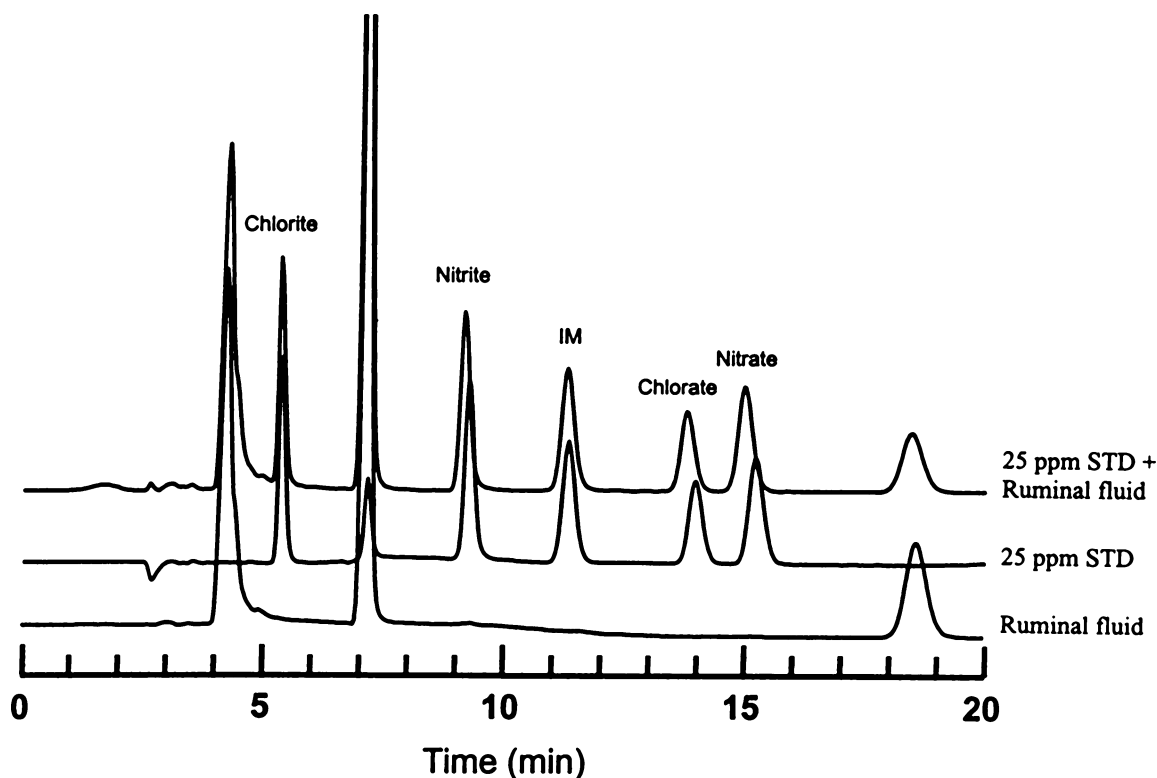


Fig. 1. HPLC tracings of 1/10 diluted ruminal fluid; the 25 ppm standard mix containing chlorate, chlorite, nitrate, nitrite, and dichloroacetate (IM); and 1/10 diluted ruminal fluid plus the 25 ppm standard mix.

the unused ruminal fluid was 7.17. All vials were incubated at 39°C on a rotating table (128 rpm). Samples (2 mL) were collected from each vial at 0, 3, 6 and 24 h of incubation for enumeration of wildtype *E. coli* via plating of 10-fold serial dilutions (in 0.4 M sodium phosphate buffer, pH 6.5) on 3M *E. coli*/Coliform Count petrifilm (3M Microbiology Products, St. Paul, MN), and for measurement of chlorate concentrations as described below.

Standard curve for disappearance of chlorate in ruminal fluid

The standard curve for chlorate disappearance was constructed over the range of 25–150 ppm chlorate ion concentration. The standard curve was generated by adding chlorate, EDA (5 μ L), the IM solution (7.5 μ L), and diluted with H₂O to a total volume of 1-mL to produce standards containing 25, 50, 75, 100, and 150 ppm of chlorate ion. This series of HPLC standards was analyzed at the beginning of each sample set, and to ensure uniformity of analysis, an additional 75 ppm standard was placed after every 6th sample.

Sample preparation and calculations for disappearance of chlorate in ruminal fluid

A 125- μ L aliquot of each ruminal fluid sample was mixed with EDA (5 μ L) and IM solution (7.5 μ L) and diluted with H₂O (862.5 μ L) to a 1-mL volume. Since ruminal

fluid samples were diluted 1:8 for HPLC analysis, chlorate results were multiplied eightfold. In addition, evaporation from aerobically incubated vials at 3, 6, and 24 h was 4.21, 10.57 and 21.18%, respectively, and required adjustment for concentration by using the following correction factors: 0.9579, 0.8943, and 0.7882, respectively.

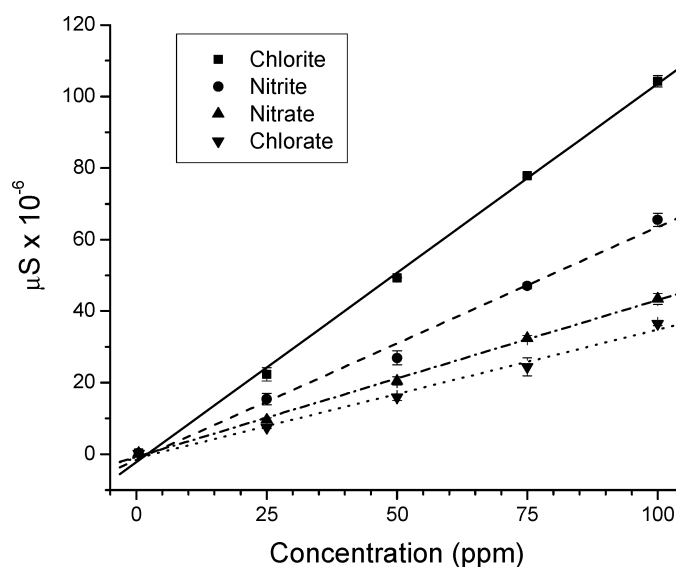


Fig. 2. Standard curves shown as micro-siemens (μ S) vs. concentration for chlorite, nitrite, nitrate, and chlorate. The error bars show the \pm standard deviation of 4 determinations.

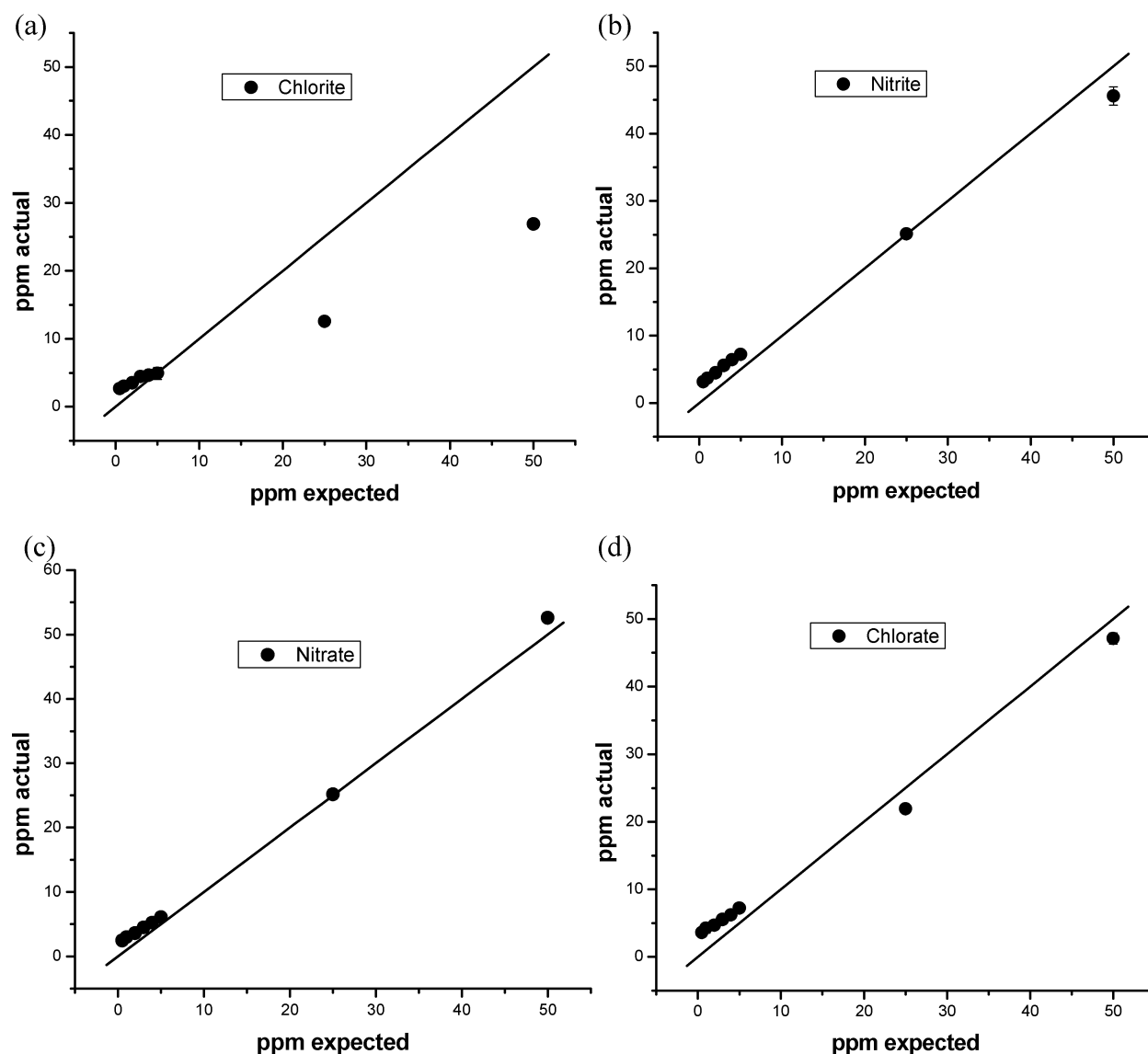


Fig. 3. A comparison of the actual level of (a) chlorite, (b) nitrite, (c) nitrate, and (d) chlorate observed in spiked 1/10 diluted ruminal fluid to the theoretical expected level. The straight lines are the theoretical expected levels in the spiked ruminal fluid. The error bars show \pm standard deviation of the mean of three determinations.

Concentrations of chlorate and \log_{10} transformations of wildtype *E. coli* concentrations were analyzed for main effects of chlorate, reductant, aerobic or anaerobic atmosphere and their interactions as specified using a repeated measures analysis of variance (Statistix[®] 8 Analytical Software, Tallahassee, FL, USA). The net change values of the *E. coli* concentrations determined after 6 h of incubation were analyzed for main effects of chlorate, reductant, and their interactions by analysis of variance with a Tukey's separation of means.

Results and discussion

The HPLC tracing of a standard mixture containing 25 ppm each of chlorite, nitrite, chlorate, nitrate, and dichloroac-

etate (the internal marker (IM)) is shown in Figure 1. Figure 1 also shows an HPLC tracing of a 1/10 dilution of ruminal fluid, and an HPLC tracing of a 1/10 dilution of ruminal fluid containing 25 ppm of chlorite, nitrite, chlorate, and nitrate ions. All of the ions, except for chlorite, appear to be well resolved. The chlorite ion is positioned in the chromatogram just within the tail of the unidentified preceding peak at 4.2 min. The retention times for chlorite, nitrite, IM, chlorate, and nitrate are 5.3, 9.2, 11.2, 13.9, and 15.2 min., respectively. The large peak at 7.1 min. is the chloride ion.

The standard curves for chlorite, nitrite, nitrate and chlorate are shown in Figure 2. Each data point in Figure 2 is the mean \pm standard deviation of four separate determinations. The data are plotted as the detector response in

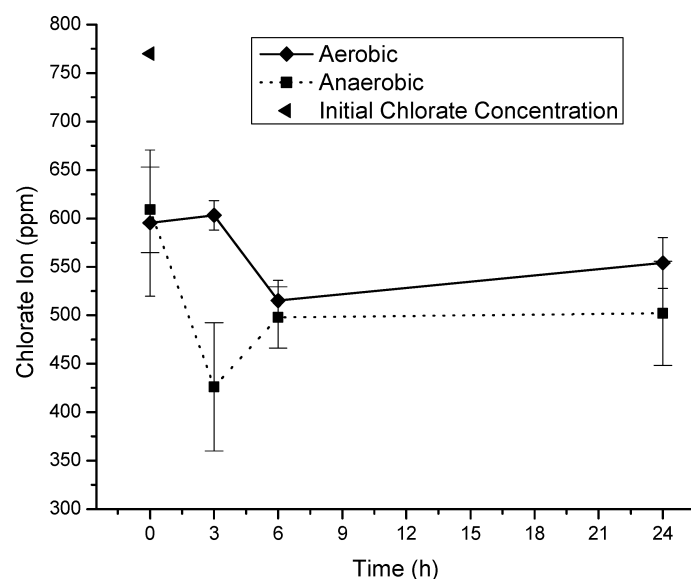
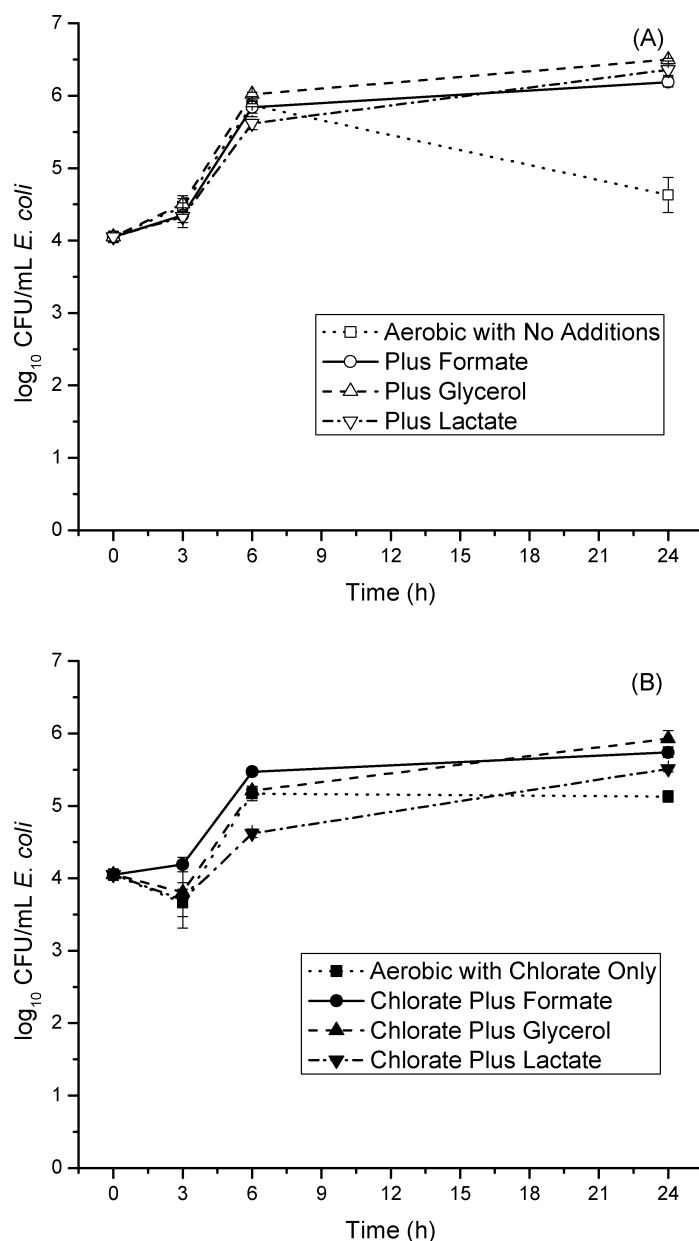
Table 1. Percent recovery of chlorite, nitrite, nitrate, and chlorate spiked in a 1/10 dilution of bovine ruminal fluid

	% Recovery			
	Concentration (ppm)			
	0.5	5	25	50
Chlorite	533.0 ± 0.2	82.9 ± 0.3	50.2 ± 0.1	53.8 ± 0.7
Nitrite	629.6 ± 7.0	139.2 ± 1.0	100.5 ± 0.5	91.2 ± 2.7
Nitrate	493.1 ± 4.1	120.2 ± 1.7	100.7 ± 0.3	105.2 ± 0.5
Chlorate	718.0 ± 4.1	147.5 ± 2.1	87.6 ± 0.5	94.2 ± 1.7

Mean values (n = 3) ± standard deviation of percent recovery.

micro-siemens (μS) vs. the concentration in ppm of each ion in the standard solution from 0.5 to 100 ppm. These are well defined linear curves with R^2 values of 0.99846, 0.99106, 0.99854, and 0.99138, for chlorite, nitrite, nitrate, and chlorate, respectively.

In the 1/10 dilution of ruminal fluid, comparison of the observed levels of the 4 spiked ions vs. the theoretically expected levels of the ions revealed that the distribution of data points for nitrite, nitrate, and chlorate follow the theoretical expected line (Fig. 3). However, the results for chlorite (Fig. 3a) were extremely low, unlike the results observed when the standards were diluted with H_2O . Chlorite may have reacted with or bound to some component(s) of the ruminal fluid, or it may have been reduced to chloride. We also have been unable to recover chlorite from chlorite-spiked raw hamburger (unpublished results). Chlorite was absent from tissues of cattle administered sodium [^{36}Cl]chlorate, while chlorate and chloride were the only radioactive chlo-

**Fig. 4.** Measurement of chlorate ion concentrations from chlorate-fortified aerobically and anaerobically incubated ruminal fluid. Sample collection times were 0, 3, 6, and 24 h.**Fig. 5.** Measurement of wildtype *E. coli* concentrations during aerobic incubation with or without added reductant in the absence (A) or presence of added chlorate (B). Values are the mean from triplicate incubations, SD are less than 0.24 \log_{10} CFU/mL unless indicated otherwise. Sample collection times were 0, 3, 6, and 24 h.

rine species present in the urine, and chloride was the major radioactive residue in edible tissues.^[27,28] Thus, chlorite is very unstable in ruminal fluid and can not be accurately determined in bovine ruminal fluid. Furthermore, observed values for all four ions at low concentrations in ruminal fluid tended to be higher than the theoretically predicted values (Fig. 3), as demonstrated by the percentage recovery for each ion (Table 1). At a concentration of 0.5 ppm, recovery was several-fold above 100% for all ions. As the concentration increased the percent recovery for ions other

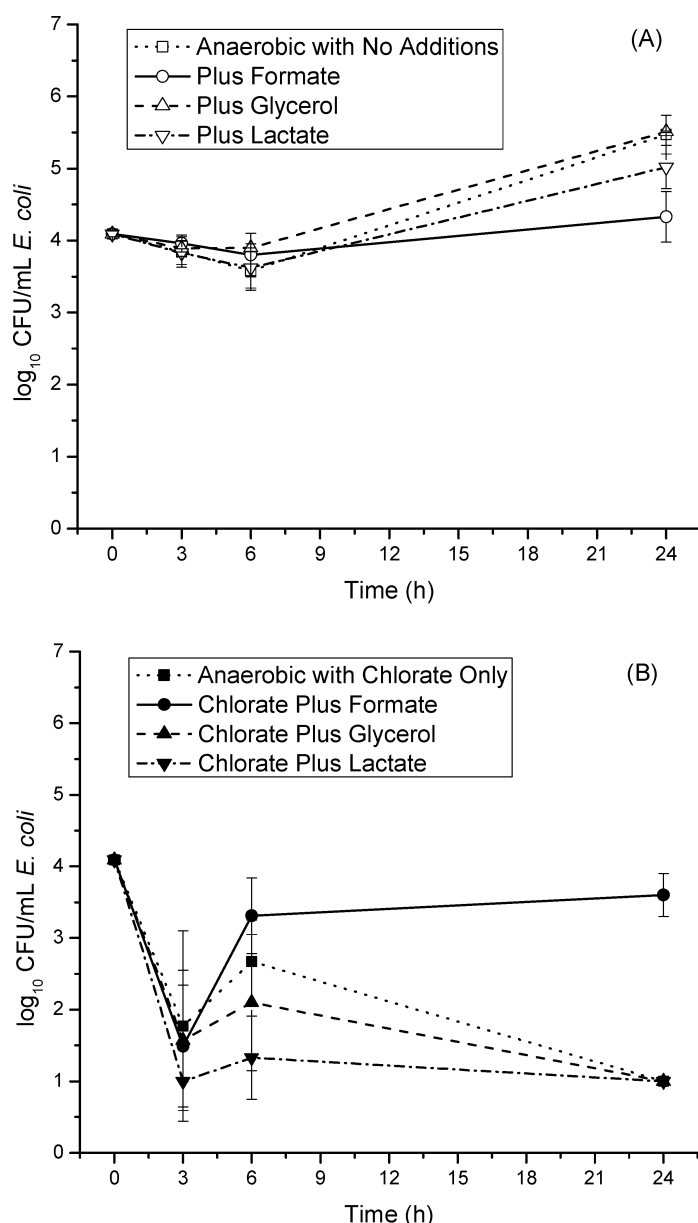


Fig. 6. Measurement of wildtype *E. coli* concentrations during anaerobic incubation with or without added reductant in the absence (A) or presence of added chlorate (B). Except for formate, *E. coli* concentrations decreased to the level of detection (10 cells/mL) by 24 h. Values are the mean from triplicate incubations, SD are less than 0.24 log₁₀ CFU/mL unless indicated otherwise. Sample collection times were 0, 3, 6, and 24 h.

than chlorite became closer to theoretical. We hypothesize that the complex nature of ruminal fluid contributes in some way to the increased values at low concentrations of anions. It is interesting that chlorite shows a recovery of 82.9% at a concentration of 5 ppm. However, this result is probably caused by at least two phenomena, the increase in the observed ion level at lower ion concentrations and the loss of

chlorite due to chlorite reacting or binding with a component(s) in ruminal fluid.

Chlorate was fortified into ruminal fluid at 770 ppm ClO_3^- to evaluate the disappearance of the ClO_3^- ion over a 24-h time period under aerobic and anaerobic (100% CO_2) conditions. Samples collected at 0, 3, 6, and 24 h of incubation were analyzed for disappearance of chlorate by HPLC over four different days using a standard curve ranging from 25 to 150 ppm, which was generated each day. The four standard curves showed consistent linearity with a mean R^2 value of 0.996973 ± 0.002816 . Figure 4 shows the comparison of the level of ClO_3^- ion in ruminal fluid kept under aerobic conditions vs. the level of ClO_3^- ion in ruminal fluid kept under anaerobic conditions. Main effects of atmosphere (aerobic versus anaerobic) ($P < 0.01$) and time ($P < 0.01$) on ClO_3^- ion concentration was observed, with concentrations being reduced in ruminal fluid more during anaerobic than aerobic incubation (Fig. 4). Main effects of time ($P < 0.01$) and an atmosphere \times time interaction ($P < 0.01$) were also observed on chlorate concentrations. Chlorate concentrations in anaerobic cultures were decreased from initial levels ($P < 0.05$) at all other sampling times, but during aerobic incubation chlorate concentrations were significantly lower ($P < 0.05$) than initial values only at 6 h, and not at 3 and 24 h (Fig. 4). Also readily apparent in the figures was that more than 100 ppm of the 770 ppm chlorate quantitatively added to each incubation at time 0 had disappeared by our first sampling time, which was within minutes of combining the ruminal fluid with chlorate. This suggests that chlorate uptake likely proceeded very quickly during both aerobic and anaerobic incubations. This finding is consistent with published reports that while nitrate uptake by the two polytopic membrane proteins NarK and NarU in *E. coli* is down-regulated by molecular oxygen, chlorate uptake occurs independent of the action of these nitrate uptake proteins and their control by oxygen.^[37,38] We observed a rebound in chlorate concentrations in anaerobically- but not aerobically-grown incubations and speculate that this may result from release of intracellular chlorate due to lysis of the anaerobically grown cells. In anaerobically grown *E. coli*, chlorate is thought to be reduced intracellularly by respiratory nitrate reductase NarG to chlorite which subsequently kills the cells,^[11,12,14] but reports on the extent of this reduction are lacking, particularly in cells that have been killed. Aerobically grown *E. coli*, however, do not reduce chlorate and are generally insensitive unless deficient in formate dehydrogenase activity.^[39] Chlorate inhibition of *E. coli* during aerobic growth in pure culture was reported by Newman *et al.*,^[40] but in that case the mechanism was not well defined. Our *E. coli* survivability curves (Figs. 5 and 6) support the concept that chlorate was bactericidal during anaerobic incubation, but only slightly inhibitory during aerobic incubation as indicated by a net increase in *E. coli* concentrations by 6 h of aerobic incubation compared to a net decrease in *E. coli* in anaerobic incubations (Table 2). Except for cultures with

Table 2. Main effects of added chlorate and reductant on net change in *E. coli* concentrations ($\Delta \log_{10}$ colony forming units) determined after 6 h incubation^a

Reductant	Aerobic atmosphere		Anaerobic (CO ₂) atmosphere	
	Without added chlorate	With added chlorate	Without added chlorate	With added chlorate
None	1.82 ^b	1.12 ^c	-0.51 ^e	-1.42 ^{e,f,g}
Formate	1.79 ^b	1.42 ^b	-0.29 ^e	-0.78 ^{e,f}
Glycerol	1.97 ^b	1.16 ^c	-0.19 ^e	-1.99 ^{f,g}
Lactate	1.57 ^c	0.57 ^d	-0.47 ^e	-2.75 ^g
Chlorate effect	$P < 0.0001$		$P < 0.0001$	
Reductant effect	$P < 0.0001$		$P = 0.0153$	
Interaction	$P < 0.0001$		$P = 0.0272$	
SEM	0.04		0.29	

^aTests for main effects of chlorate, reductant and their interaction were accomplished using a general analysis of variance with further separation of means using a Tukey's procedure.

^{b,c,d}Means ($n = 3$) with unlike superscripts differ ($P < 0.05$).

^{e,f,g}Means ($n = 3$) with unlike superscripts differ ($P < 0.05$).

added formate, all cultures supplemented with chlorate and anaerobically incubated decreased in *E. coli* to the level of detection (10 cells/mL) by 24 h.

Addition of the reductants formate, glycerol, and lactate to ruminal fluid did not alter ($P > 0.05$) chlorate disappearance in aerobic (data not shown) or anaerobic (Fig. 7) cultures, although the levels of ClO₃⁻ ion in anaerobic incubations were numerically lower at 24 h, particularly for those incubated with added sodium formate compared with anaerobic incubations without added reductant. However, we observed that when formate was added to the anaerobic

chlorate-supplemented ruminal fluid cultures, it appeared to moderate the *E. coli*-killing activity of chlorate and this moderation is likely not due to the depletion of chlorate. Formate oxidation by an anaerobically expressed formate dehydrogenase is reported to protect the respiratory chain in stationary cells of *E. coli* and *Salmonella* and to prevent its disruption by host-produced antimicrobial peptides.^[41] However, whether such a mechanism was active here is not known. Reductant by time interactions were not observed on chlorate ion concentrations ($P > 0.05$).

Conclusion

The analytical method presented here for nitrite, nitrate, and chlorate in ruminal fluid is a simple and quick method for determining the levels of these ions. Although these ions are easily observed at low concentration (0.5 ppm) in ruminal fluid, the accuracy of our assay method at low levels is extremely poor. Due to the reactivity of chlorite with ruminal fluid component(s), levels of chlorite in ruminal fluid was not accurately determined.

By using the described method of chlorate determination in ruminal fluid with a standard curve ranging from 25 to 150 ppm we were able to demonstrate a more rapid ($P < 0.01$) elimination of ClO₃⁻ ion levels in chlorate-fortified ruminal fluid during anaerobic than during aerobic incubation conditions. Also, in chlorate-fortified anaerobically-incubated ruminal fluid, addition of the reductant formate appeared to decrease 24-h chlorate concentrations compared with incubations without formate; however, the addition of sodium formate appeared to moderate the bactericidal effect of chlorate against *E. coli*.

This work demonstrates that chlorate fortification of ruminal fluid results in the killing of *E. coli* bacteria under anaerobic conditions. Based upon the suggestive results

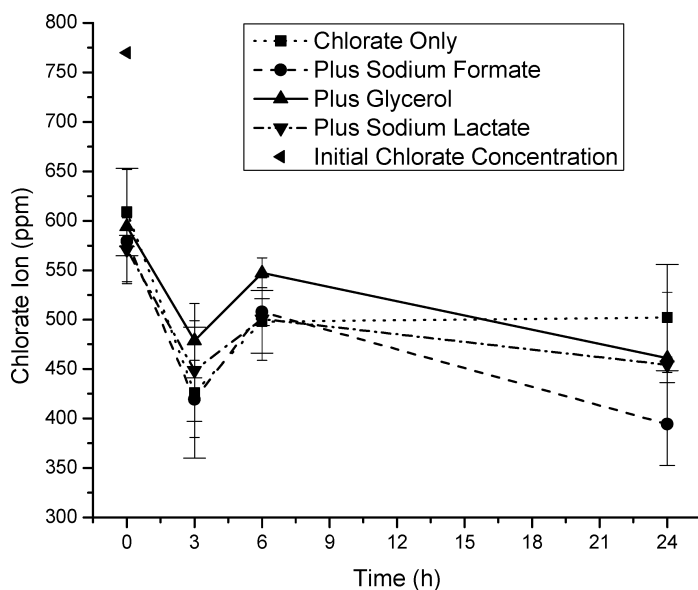


Fig. 7. Measurement of chlorate ion concentrations during anaerobic incubation of ruminal fluid over a 24-h period fortified with chlorate and the reductants, formate, glycerol and lactate. Sample collection times were 0, 3, 6, and 24 h.

obtained with the addition of the reductants glycerol and lactate to chlorate-fortified ruminal fluid, further studies probing how these reductants are involved in decreasing *E. coli* are warranted. The addition of reductants in chlorate treatments may be necessary, particularly when reducing equivalents may be limiting, such as when cattle are subjected to an extended transit or during a fast, or during extended composting of cow manure for use in produce production. The applications of chlorate treatment to aid in decontamination and on-farm pathogen reduction strategies are being investigated in animals. However, an investigation using chlorate to reduce the pathogen levels in waste holding reservoirs like swine waste lagoons or composting systems required for intense animal production is suggested.

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